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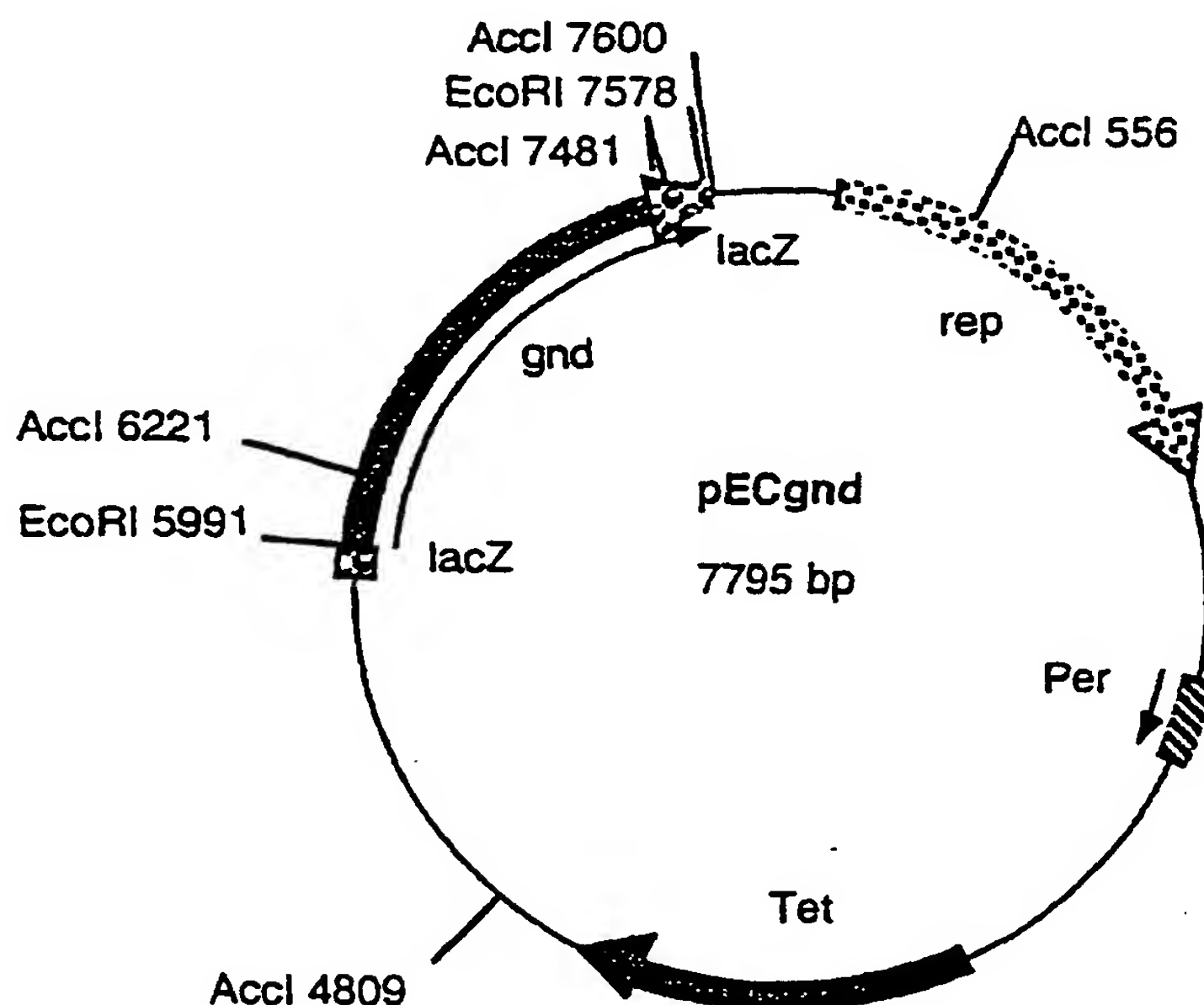
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(54) Title: PROCESS FOR THE FERMENTATIVE PREPARATION OF L-AMINO ACIDS WITH AMPLIFICATION OF THE GND GENE

Map of the plasmid pECgnd



(57) Abstract: The invention relates to a process for the preparation of L-amino acids by fermentation of coryneform bacteria, which comprises carrying out the following steps: a) fermentation of the desired L-amino acid-producing bacteria in which at least the gnd gene is amplified, b) concentration of the L-amino acid in the medium or in the cells of the bacteria and c) isolation of the L-amino acid produced.

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

**Process for the fermentative preparation of L-amino acids
with amplification of the gnd gene**

The invention relates to a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, using coryneform bacteria in which at least the gnd gene is amplified.

Prior art

L-Amino acids are used in animal nutrition, in human medicine and in the pharmaceuticals industry.

10 It is known that amino acids are prepared by fermentation from strains of coryneform bacteria, in particular *Corynebacterium glutamicum*. Because of their great importance, work is constantly being undertaken to improve the preparation processes. Improvements to the process can
15 relate to fermentation measures, such as e.g. stirring and supply of oxygen, or the composition of the nutrient media, such as e.g. the sugar concentration during the fermentation, or the working up to the product form by e.g. ion exchange chromatography, or the intrinsic output
20 properties of the microorganism itself.

Methods of mutagenesis, selection and mutant selection are used to improve the output properties of these microorganisms. Strains which are resistant to antimetabolites, such as e.g. the threonine analogue α -
25 amino- β -hydroxyvaleric acid (AHV), or are auxotrophic for metabolites of regulatory importance and produce L-amino acids such as e.g. threonine are obtained in this manner.

Methods of the recombinant DNA technique have also been employed for some years for improving the strain of
30 *Corynebacterium glutamicum* strains which produce L-amino acids.

Object of the invention

The inventors had the object of providing improved processes for the fermentative preparation of L-amino acids with coryneform bacteria.

5 Description of the invention

L-Amino acids are used in human medicine and in the pharmaceuticals industry, in the foodstuffs industry and especially in animal nutrition. There is therefore a general interest in providing new improved processes for
10 the preparation of amino acids.

The invention provides a process for the fermentative preparation of L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, using coryneform bacteria in which the nucleotide sequence which codes for
15 the enzyme 6-phosphogluconate dehydrogenase (EC number 1.1.1.44) (gnd gene) is amplified, in particular over-expressed.

The strains employed preferably already produce L-amino acids before amplification of the gnd gene.

20 Preferred embodiments are to be found in the claims.

The term "amplification" in this connection describes the increase in the intracellular activity of one or more enzymes in a microorganism which are coded by the corresponding DNA, for example by increasing the number of
25 copies of the gene or genes, using a potent promoter or using a gene which codes for a corresponding enzyme having a high activity, and optionally combining these measures.

The microorganisms which the present invention provides can prepare L-amino acids from glucose, sucrose, lactose,
30 fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of

coryneform bacteria, in particular of the genus
Corynebacterium. Of the genus Corynebacterium, there may be
mentioned in particular the species Corynebacterium
glutamicum, which is known among experts for its ability to
5 produce L-amino acids.

Suitable strains of the genus Corynebacterium, in
particular of the species Corynebacterium glutamicum, are,
for example, the known wild-type strains

Corynebacterium glutamicum ATCC13032
10 Corynebacterium acetoglutamicum ATCC15806
Corynebacterium acetoacidophilum ATCC13870
Corynebacterium thermoaminogenes FERM BP-1539
Brevibacterium flavum ATCC14067
Brevibacterium lactofermentum ATCC13869
15 Brevibacterium divaricatum ATCC14020

and L-amino acid-producing mutants prepared therefrom,

such as, for example, the L-threonine-producing strains

Corynebacterium glutamicum ATCC21649
Brevibacterium flavum BB69
20 Brevibacterium flavum DSM5399
Brevibacterium lactofermentum FERM-BP 269
Brevibacterium lactofermentum TBB-10

and such as, for example, the L-isoleucine-producing
strains

25 Corynebacterium glutamicum ATCC 14309
Corynebacterium glutamicum ATCC 14310
Corynebacterium glutamicum ATCC 14311
Corynebacterium glutamicum ATCC 15168
Corynebacterium ammoniagenes ATCC 6871

30 and such as, for example, the L-tryptophan-producing
strains

Corynebacterium glutamicum ATCC21850
Corynebacterium glutamicum KY9218 (pKW9901)

and such as, for example, the L-lysine-producing strains
Corynebacterium glutamicum FERM-P 1709
Brevibacterium flavum FERM-P 1708
Brevibacterium lactofermentum FERM-P 1712
5 Corynebacterium glutamicum FERM-P 6463
Corynebacterium glutamicum FERM-P 6464
Corynebacterium glutamicum DSM5715
Corynebacterium glutamicum DM58-1
Corynebacterium glutamicum DSM12866.

10 It has been found that coryneform bacteria produce L-amino acids, in particular L-lysine, L-threonine, L-isoleucine and L-tryptophan, in an improved manner after over-expression of the gnd gene which codes for 6-phosphogluconate dehydrogenase (EC number 1.1.1.44).

15 The gnd gene codes for the enzyme 6-phosphogluconate dehydrogenase, which catalyses the oxidative decarboxylation of 6-phosphogluconic acid to ribulose 5-phosphate. The nucleotide sequence of the gnd gene is disclosed in JP-A-9-224662. The gnd gene described in the
20 text reference mentioned is used according to the invention for the first time. Alleles of the gnd gene which result from the degeneracy of the genetic code or due to sense mutations of neutral function can furthermore be used.

To achieve an amplification (e.g. over-expression), the
25 number of copies of the corresponding genes is increased, or the promoter and regulation region or the ribosome binding site upstream of the structural gene is mutated. Expression cassettes which are incorporated upstream of the structural gene act in the same way. By inducible
30 promoters, it is additionally possible to increase the expression in the course of fermentative L-amino acid formation. The expression is likewise improved by measures to prolong the life of the m-RNA. Furthermore, the enzyme activity is also increased by preventing the degradation of
35 the enzyme protein. The genes or gene constructs are either

present here in plasmids with a varying number of copies, or are integrated and amplified in the chromosome. Alternatively, an over-expression of the genes in question can furthermore be achieved by changing the composition of the media and the culture procedure.

Instructions in this context can be found by the expert, inter alia, in Martin et al. (Bio/Technology 5, 137-146 (1987)), in Guerrero et al. (Gene 138, 35-41 (1994)), Tsuchiya and Morinaga (Bio/Technology 6, 428-430 (1988)),
10 in Eikmanns et al. (Gene 102, 93-98 (1991)), in European Patent Specification EPS 0 472 869, in US Patent 4,601,893, in Schwarzer and Pühler (Bio/Technology 9, 84-87 (1991), in Reinscheid et al. (Applied and Environmental Microbiology 60, 126-132 (1994)), in LaBarre et al. (Journal of
15 Bacteriology 175, 1001-1007 (1993)), in Patent Application WO 96/15246, in Malumbres et al. (Gene 134, 15-24 (1993)), in Japanese Laid-Open Specification JP-A-10-229891, in Jensen and Hammer (Biotechnology and Bioengineering 58, 191-195 (1998)) and in known textbooks of genetics and
20 molecular biology.

By way of example, 6-phosphogluconate dehydrogenase was over-expressed with the aid of a plasmid. The *E. coli* - *C. glutamicum* shuttle vector pEC-T18mob2 shown in Figure 1 was used for this. After incorporation of the *gnd* gene into the
25 *EcoRI* cleavage site of pEC-T18mob2, the plasmid pECgnd shown in Figure 2 was formed.

Other plasmid vectors which are capable of replication in *C. glutamicum*, such as e.g. pEKEx1 (Eikmanns et al., Gene 102:93-98 (1991)) or pZ8-1 (EP-B- 0 375 889), can be used
30 in the same way.

In addition, it may be advantageous for the production of L-amino acids to amplify one or more enzymes of the particular biosynthesis pathway, of glycolysis, of anaplerosis, of the pentose phosphate pathway or of amino

acid export, in addition to amplification of the gnd gene which codes for 6-phosphogluconate dehydrogenase.

Thus, for example, in particular for the preparation of L-threonine, one or more genes chosen from the group

5 consisting of

- the hom gene which codes for homoserine dehydrogenase (Peoples et al., Molecular Microbiology 2, 63-72 (1988)) or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase (Archer et al., Gene
10 107, 53-59 (1991),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns et al., Journal of Bacteriology 174: 6076-6086 (1992)),
- the pyc gene which codes for pyruvate carboxylase
15 (Peters-Wendisch et al., Microbiology 144: 915-927 (1998)),
- the mqo gene which codes for malate:quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- 20 • the tkt gene which codes for transketolase (accession number AB023377 of the databank of European Molecular Biology Laboratories (EMBL, Heidelberg, Germany)),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- 25 • the thrE gene which codes for threonine export (DE 199 41 478.5; DSM 12840),
- the zwf gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE: 199 41 478.5)

can be amplified, in particular over-expressed, at the same time.

Thus, for example, in particular for the preparation of L-lysine, one or more genes chosen from the group consisting of

- the dapA gene which codes for dihydrodipicolinate synthase (EP-B 0 197 335),
- a lysC gene which codes for a feed back resistant aspartate kinase (Kalinowski et al. (1990), Molecular and General Genetics 224: 317-324),
- the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the pyc gene which codes for pyruvate carboxylase (Eikmanns (1992), Journal of Bacteriology 174:6076-6086),
- the mqo gene which codes for malate-quinone oxidoreductase (Molenaar et al., European Journal of Biochemistry 254, 395-403 (1998)),
- the tkt gene which codes for transketolase (accession number AB023377 of the databank of European Molecular Biologies Laboratories (EMBL, Heidelberg, Germany)),
- the zwf gene which codes for glucose 6-phosphate dehydrogenase (JP-A-09224661),
- the lysE gene which codes for lysine export (DE-A-195 48 222),
- the zwal gene (DE 199 59 328.0; DSM 13115),
- the eno gene which codes for enolase (DE 199 47 791.4)

can be amplified, in particular over-expressed, at the same time.

It may furthermore be advantageous for the production of L-amino acids at the same time to attenuate one or more of
5 the genes chosen from the group consisting of:

- the pck gene which codes for phosphoenol pyruvate carboxykinase (DE 199 50 409.1; DSM 13047),
- the pgi gene which codes for glucose 6-phosphate isomerase (US 09/396,478, DSM 12969),
- 10 • the poxB gene which codes for pyruvate oxidase (DE 199 51 975.7; DSM 13114),
- the zwa2 gene (DE: 199 59 327.2; DSM 13113)

in addition to the amplification of the gnd gene.

In addition to over-expression of 6-phosphogluconate
15 dehydrogenase, it may furthermore be advantageous for the production of L-amino acids to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London,
20 UK, 1982).

The microorganisms prepared according to the invention can be cultured continuously or discontinuously in the batch process (batch culture) or in the fed batch (feed process) or repeated fed batch process (repetitive feed process) for
25 the purpose of L-amino acid production. A summary of known culture methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess Technology 1. Introduction to Bioprocess Technology (Gustav Fischer Verlag, Stuttgart, 30 1991)) or in the textbook by Storhas (Bioreaktoren und

periphere Einrichtungen [Bioreactors and Peripheral Equipment] (Vieweg Verlag, Braunschweig/Wiesbaden, 1994)).

The culture medium to be used must meet the requirements of the particular microorganisms in a suitable manner.

- 5 Descriptions of culture media for various microorganisms are contained in the handbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981). Sugars and carbohydrates, such as e.g. glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose, oils and fats, such as e.g. soya oil, sunflower oil, groundnut oil and coconut fat, fatty acids, such as e.g. palmitic acid, stearic acid and linoleic acid, alcohols, such as e.g. glycerol and ethanol, and organic acids, such as e.g. acetic acid, can be used as the source of carbon. These substances can be used individually or as a mixture. Organic nitrogen-containing compounds, such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soya bean flour and urea, or inorganic compounds, such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate, can be used as the source of nitrogen. The sources of nitrogen can be used individually or as a mixture. Potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts can be used as the source of phosphorus. The culture medium must furthermore comprise salts of metals, such as e.g. magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances, such as amino acids and vitamins, can be employed in addition to the above-mentioned substances. Suitable precursors can moreover be added to the culture medium. The starting substances mentioned can be added to the culture in the form of a single batch, or can be fed in during the culture in a suitable manner.

- Basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be employed in a suitable manner to control the pH. Antifoams, such as e.g. fatty acid polyglycol esters, can be employed to control the development of foam. Suitable substances having a selective action, e.g. antibiotics, can be added to the medium to maintain the stability of plasmids. To maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as e.g. air, are introduced into the culture. The temperature of the culture is usually 20°C to 45°C, and preferably 25°C to 40°C. Culturing is continued until a maximum of L-amino acid has formed. This target is usually reached within 10 hours to 160 hours.
- 15 The analysis of L-amino acids can be carried out by anion exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman et al. (Analytical Chemistry, 30, (1958), 1190), or it can take place by reversed phase HPLC as described by Lindroth et al. (Analytical Chemistry (1979) 51:. 1167-1174).

The following microorganism has been deposited at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty:

Escherichia coli K-12 DH5 α /pEC-T18mob2 as DSM 13244

The following figures are attached:

- Figure 1: Map of the plasmid pEC-T18mob2
- Figure 2: Map of the plasmid pECgnd
- Figure 3: Map of the plasmid pBGNA
- 5 • Figure 4: Map of the plasmid pCR2.1poxBint

The base pair numbers stated are approx. values obtained in the context of reproducibility.

The abbreviations used have the following meaning:

Re Figure 1:

- | | | |
|----|-------------|---|
| 10 | Tet: | Resistance gene for tetracycline |
| | oriV: | Plasmid-coded replication origin of E. coli |
| | RP4mob: | mob region for mobilizing the plasmid |
| | rep: | Plasmid-coded replication origin from C. glutamicum plasmid pGA1 |
| 15 | per: | Gene for controlling the number of copies from pGA1 |
| | lacZ-alpha: | lacZ α gene fragment (N-terminus) of the β -Galactosidase gene |

Re Figure 2:

- | | | |
|----|------|--|
| 20 | Tet: | Resistance gene for tetracycline |
| | rep: | Plasmid-coded replication origin from C. glutamicum plasmid pGA1 |
| | per: | Gene for controlling the number of copies from pGA1 |
| 25 | lacZ | Cloning relict of the lacZ α gene fragment from pEC-T18mob2 |
| | gnd: | 6-Phosphogluconate dehydrogenase gene |

Re Figure 3:

- | | | |
|----|--------|---|
| | LacP: | Promoter of the E. coli lactose operon |
| 30 | CMV: | Promoter of cytomegalovirus |
| | ColE1: | Replication origin of the plasmid ColE1 |

TkpolyA: Polyadenylation site
Kan r: Kanamycin resistance gene
SV40ori: Replication origin of Simian virus 40
gnd: 6-Phosphogluconate dehydrogenase gene

5 Re Figure 4:

ColE1 ori: Replication origin of the plasmid ColE1
lacZ: Cloning relict of the lacZ α gene fragment
fl ori: Replication origin of phage fl
KmR: Kanamycin resistance
10 ApR: Ampicillin resistance
poxBint: internal fragment of the poxB gene

Moreover, the following abbreviations have been used:

AccI: Cleavage site of the restriction enzyme AccI
BamHI: Cleavage site of the restriction enzyme BamHI
15 EcoRI: Cleavage site of the restriction enzyme EcoRI
HindIII: Cleavage site of the restriction enzyme HindIII
KpnI: Cleavage site of the restriction enzyme KpnI
PstI: Cleavage site of the restriction enzyme PstI
PvuI: Cleavage site of the restriction enzyme PvuI
20 SalI: Cleavage site of the restriction enzyme SalI
SacI: Cleavage site of the restriction enzyme SacI
SmaI: Cleavage site of the restriction enzyme SmaI
SphI: Cleavage site of the restriction enzyme SphI
XbaI: Cleavage site of the restriction enzyme XbaI
25 XhoI: Cleavage site of the restriction enzyme XhoI

Examples

The following examples will further illustrate this invention. The molecular biology techniques, e.g. plasmid DNA isolation, restriction enzyme treatment, ligations, standard transformations of *Escherichia coli* etc. used are, (unless stated otherwise), described by Sambrook et al., (Molecular Cloning. A Laboratory Manual (1989) Cold Spring Harbour Laboratories, USA).

Example 1

- 10 Construction of a gene library of *Corynebacterium glutamicum* strain AS019

A DNA library of *Corynebacterium glutamicum* strain AS019 (Yoshihama et al., Journal of Bacteriology 162, 591-597 (1985)) was constructed using *ẽ* Zap ExpressTM system, (Short et al., (1988) Nucleic Acids Research 16: 7583-7600), as described by O'Donohue (O'Donohue, M. (1997). The Cloning and Molecular Analysis of Four Common Aromatic Amino Acid Biosynthetic Genes from *Corynebacterium glutamicum*. Ph.D. Thesis, National University of Ireland, Galway). *ẽ* Zap ExpressTM kit was purchased from Stratagene (Stratagene, 11011 North Torrey Pines Rd., La Jolla, California 92037) and used according to the manufacturers instructions. AS019-DNA was digested with restriction enzyme *Sau*3A and ligated to *Bam*HI treated and dephosphorylated *ẽ* Zap ExpressTM arms.

Example 2

Cloning and sequencing of the *gnd* gene

1. Construction of a *gnd* probe

A radiolabelled oligonucleotide, internal to the *gnd* gene, was used to probe the AS019 *ẽ* Zap ExpressTM library described above. The oligonucleotide was produced using degenerate PCR primers internal to the *gnd* gene. The

degenerate nucleotide primers designed for the PCR amplification of gnd DNA fragments were as follows:

gnd1: 5' ATG GTK CAC ACY GGY ATY GAR TA 3'

gnd2: 5' RGT CCA YTT RCC RGT RCC YTT 3'

5 with R=A+G; Y=C+T; K=T+G.

The estimated size of the resulting PCR product was 252 bp approximately.

Optimal PCR conditions were determined to be as follows:

35 cycles
10 94°C for 1 minute
55°C for 1 minute
72°C for 30 seconds
2.5 - 3.5 mM MgCl₂
100 - 150 ng AS019 genomic DNA

15 Sequence analysis of the resulting PCR product confirmed the product to be an internal portion of a gnd gene. Sequence analysis was carried out using the universal forward and reverse primers, and T7 sequencing kit from Pharmacia Biotech, (St. Albans, Herts, UK). The sequence of
20 the PCR product is shown in SEQ ID No. 1.

2. Cloning

Screening of the AS019 ÷ Zap ExpressTM library was carried out according to the ÷ Zap ExpressTM system protocol, (Stratagene, 11011 North Torrey Pines Rd., La Jolla,
25 California 92037). Southern Blot analysis was then carried out on isolated clones. Southern transfer of DNA was as described in the Schleicher and Schuell protocols manual employing NytranTM as membrane („Nytran, Modified Nylon-66 Membrane Filters" (March 1987), Schleicher and Schuell,
30 Dassel, Germany). Double stranded DNA fragments, generated using the same primers and optimal PCR conditions as described above, were radiolabelled with α -³²P-dCTP using

the MultiprimeTM DNA labelling kit from Amersham Life Science (Amersham Pharmacia Biotech UK Limited, Little Chalfont, Buckinghamshire, UK) according to the manufacturers instructions. Prehybridisation, hybridization
5 and washing conditions were as described in the Schleicher and Schuell protocols manual. Autoradiography was carried out according to the procedure outlined in the handbook of Sambrook et al. using AgFa Curix RPIL film. Thus several
10 gnd clones were identified. Plasmid DNA was isolated from one of the clones, designated pBGNA (Figure 3) and chosen for further analysis.

3. Sequencing

The Sanger Dideoxy chain termination method of Sanger et al. (Proceedings of the National Academy of Sciences USA
15 74, 5463-5467 (1977)) was used to sequence the cloned insert of pBGNA. The method was applied using the T7 sequencing kit and α -³⁵S-dCTP from Pharmacia Biotech (St. Albans, Herts, UK). Samples were electrophoresed for 3-8
20 hours on 6% polyacrylamide/urea gels in TBE buffer at a constant current of 50 mA, according to the Pharmacia cloning and sequencing instructions manual („T7 SequencingTM Kit“, ref.XY-010-00-19, Pharmacia Biotech, 1994). Sequence
25 analysis was carried out using internal primers designed from the sequence known of the internal gnd PCR product (SEQ ID NO 1) allowing the entire gnd gene sequence to be deduced.

The sequences of the internal primers were as follows:

Internal primer 1: 5' GGT GGA TGC TGA AAC CG 3'
Internal primer 2: 5' GCT GCA TGC CTG CTG CG 3'
30 Internal primer 3: 5' TTG TTG CTT ACG CAC AG 3'
Internal primer 4: 5' TCG TAG GAC TTT GCT GG 3'

Sequence obtained was then analyzed using the DNA Strider programme, (Marck (1988), Nucleic Acids Research 16: 1829-1836), version 1.0 on an Apple Macintosh computer. This

program allowed for analyses such as restriction site usage, open reading frame analysis and codon usage determination. Searches between DNA sequence obtained and those in EMBL and Genbank databases were achieved using the BLAST programme (Altschul et al., (1997), Nucleic Acids Research 25: 3389-3402). DNA and protein sequences were aligned using the Clustal V and Clustal W programs (Higgins and Sharp, 1988 Gene 73: 237-244).

The sequence thus obtained is shown in SEQ ID NO 2. The analysis of the nucleotide sequence obtained revealed an open reading frame of 1377 base pairs which was designated as gnd gene. It codes for a protein of 459 amino acids shown in SEQ ID NO 3.

Example 3

15 Preparation of the shuttle vector pEC-T18mob2

The E. coli - C. glutamicum shuttle vector pEC-T18mob2 was constructed according to the prior art.

The vector contains the replication region rep of the plasmid pGA1 including the replication effector per (US-A- 5,175,108; Nesvera et al., Journal of Bacteriology 179, 1525-1532 (1997)), the tetracycline resistance-imparting tetA(2) gene of the plasmid pAG1 (US-A- 5,158,891; gene library entry at the National Center for Biotechnology Information (NCBI, Bethesda, MD, USA) with accession number AF121000), the replication region oriV of the plasmid pMB1 (Sutcliffe, Cold Spring Harbor Symposium on Quantitative Biology 43, 77-90 (1979)), the lacZ gene fragment including the lac promoter and a multiple cloning site (mcs) (Norranders et al. Gene 26, 101-106 (1983)) and the mob region of the plasmid RP4 (Simon et al., (1983) Bio/Technology 1:784-791).

The vector constructed was transformed in the E. coli strain DH5 α (Hanahan, In: DNA cloning. A practical

approach. Vol. I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. 5 Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., USA, 1989), which had been supplemented with 5 mg/l tetracycline. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme 10 EcoRI and HindIII subsequent agarose gel electrophoresis (0.8%).

The plasmid was called pEC-T18mob2 and is shown in Figure 1. It is deposited in the form of the strain *Escherichia coli* K-12 strain DH5 α /pEC-T18mob2 at the Deutsche Sammlung 15 für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) as DSM 13244.

Example 4

Cloning of the *gnd* gene into the *E. coli* - *C. glutamicum* 20 shuttle vector pEC-T18mob2

PCR was used to amplify DNA fragments containing the entire *gnd* gene of *C. glutamicum* and flanking upstream and downstream regions using pBGNA as template. PCR reactions were carried out using oligonucleotide primers designed 25 from SEQ ID NO 2. The primers used were:

gnd fwd. primer: 5' ACT CTA GTC GGC CTA AAA TGG 3'
gnd rev. primer: 5' CAC ACA GGA AAC AGA TAT GAC 3'

PCR parameters were as follows:

35 cycles
95°C for 6 minutes
94°C for 1 minute
50°C for 1 minute
5 72°C for 45 seconds
1 mM MgCl₂
approx. 150-200ng pBGNA-DNA as template.

The PCR product obtained was cloned into the commercially available pGEM-T vector purchased from Promega Corp. (pGEM-T Easy Vector System 1, cat. no. A1360, Promega UK, Southampton) using *E. coli* strain JM109 (Yanisch-Perron et al. Gene, 33: 103-119 (1985)) as a host. The entire *gnd* gene was subsequently isolated from the pGEM T-vector on an EcoRI fragment and cloned into the *lacZ* EcoRI site of the *E. coli* - *C. glutamicum* shuttle vector pEC-T18mob2 (Figure 1), and designated pECgnd (Figure 2). Restriction enzyme analysis with AccI (Boehringer Mannheim GmbH, Germany) revealed the correct orientation (i. e. downstream the *lac*-Promotor) of the *gnd* gene in the *lacZα* gene of pEC-T18mob2.

20 Example 5

Preparation of amino acid producers with amplified 6-phosphogluconate dehydrogenase

Plasmid pECgnd from Example 3 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in the strains *Corynebacterium glutamicum* DSM 5399 and DSM 5714. The strain DSM 5399 is a threonine producer described in EP-B-0358940. The strain DSM 5714 is a lysine producer described in EP-B-0435132. Selection of transformants was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with

25 mg/l kanamycin. The strains DSM5399/pECgnd and DSM5714/pECgnd were formed in this manner.

Example 6

Preparation of threonine

- 5 The *C. glutamicum* strain DSM5399/pECgnd obtained in Example 5 was cultured in a nutrient medium suitable for the production of threonine and the threonine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate
10 with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). Brain-heart broth (Merck, Darmstadt, Germany) was used as the medium for the
15 preculture. Tetracycline (5 mg/l) was added to this medium. The preculture was incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main culture was 0.1. The medium MM-threonine was used
20 for the main culture.

Medium MM-threonine:

| | |
|---|----------|
| CSL | 5 g/l |
| MOPS | 20 g/l |
| Glucose (autoclaved separately) | 50g/l |
| Salts: | |
| (NH ₄) ₂ SO ₄ | 25 g/l |
| KH ₂ PO ₄ | 0.1 g/l |
| MgSO ₄ * 7 H ₂ O | 1.0 g/l |
| CaCl ₂ * 2 H ₂ O | 10 mg/l |
| FeSO ₄ * 7 H ₂ O | 10 mg/l |
| MnSO ₄ * H ₂ O | 5.0mg/l |
| Biotin (sterile-filtered) | 0.3 mg/l |
| Thiamine * HCl (sterile-filtered) | 0.2 mg/l |
| CaCO ₃ | 25 g/l |

The CSL (corn steep liquor), MOPS (morpholinopropanesulfonic acid) and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO₃ autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The concentration of threonine formed was determined with an amino acid analyzer from
5 Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 1.

Table 1

| Strain | OD(660) | L-Threonin g/l |
|----------------|---------|-------------------|
| DSM5399/pECgnd | 11.9 | 1.29 |
| DSM5399 | 11.8 | 0.33 |

10

Example 7

Preparation of lysine

The C. glutamicum strain DSM5714/pECgnd obtained in Example 5 was cultured in a nutrient medium suitable for the
15 production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting from
20 this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The complete medium CgIII was used as the medium for the preculture.

Medium Cg III

NaCl 2.5 g/l

Bacto-Peptide 10 g/l

Bacto-Yeast extract 10 g/l

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) was added to this medium. The preculture was incubated for 24 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded from this preculture such that the initial OD (660nm) of the main
5 culture was 0.05. Medium MM was used for the main culture.

Medium MM

| | |
|---|----------|
| CSL (corn steep liquor) | 5 g/l |
| MOPS (morpholinopropanesulfonic acid) | 20 g/l |
| Glucose (autoclaved separately) | 50g/l |
| $(\text{NH}_4)_2\text{SO}_4$ | |
| KH_2PO_4 | 25 g/l |
| $\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$ | 0.1 g/l |
| $\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$ | 1.0 g/l |
| $\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$ | 10 mg/l |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ | 10 mg/l |
| Biotin (sterile-filtered) | 0.3 mg/l |
| Thiamine * HCl (sterile-filtered) | 0.2 mg/l |
| L-Leucine (sterile-filtered) | 0.1 g/l |
| CaCO_3 | 25 g/l |

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) was added. Culturing was carried out at 33°C and 80% atmospheric humidity.

After 48 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, München). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-
5 BioTronik (Hamburg, Germany) by ion exchange chromatography and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 2.

Table 2

| Strain | OD(660) | Lysine HCl g/l |
|----------------|---------|-------------------|
| DSM5715/pECgnd | 7.7 | 14.7 |
| DSM5715 | 7.1 | 13.7 |

10 Example 8

Preparation of a genomic cosmid gene library from
Corynebacterium glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described by Tauch et al., (1995, Plasmid
15 33:168-179), and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Code no. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product
20 Description SAP, Code no. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl et al. (1987) Proceedings of the National Academy of Sciences USA 84:2160-2164), obtained from Stratagene (La Jolla, USA, Product Description SuperCos1 Cosmid Vektor Kit, Code no. 251301) was cleaved
25 with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, Product Description XbaI, Code no. 27-

0948-02) and likewise dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was then cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Code no. 27-0868-04).

5 The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch was treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, Product Description T4-DNA-Ligase, Code no.27-0870-04). The ligation mixture was then packed in phages with the aid of

10 Gigapack II XL Packing Extracts (Stratagene, La Jolla, USA, Product Description Gigapack II XL Packing Extract, Code no. 200217). For infection of the E. coli strain NM554 (Raleigh et al. 1988, Nucleic Acid Research 16:1563-1575) the cells were taken up in 10 mM MgSO₄ and mixed with an

15 aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the cells being plated out on LB agar (Lennox, 1955, Virology 1:190) + 100 µg/ml

20 ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 9

Isolation and sequencing of the poxB gene

The cosmid DNA of an individual colony (Example 8) was

25 isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-

30 02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, the cosmid fragments in the size range of 1500 to 2000 bp were

35 isolated with the QiaExII Gel Extraction Kit (Product No.

20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from Invitrogen (Groningen, Holland, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook et al. (1989, Molecular Cloning: A laboratory Manual, Cold Spring Harbor), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was then electroporated (Tauch et al. 1994, FEMS Microbiol Letters, 123:343-7) into the E. coli strain DH5 α MCR (Grant, 1990, Proceedings of the National Academy of Sciences U.S.A., 87:4645-4649) and plated out on LB agar (Lennox, 1955, Virology, 1:190) with 50 μ g/ml zeocin. The plasmid preparation of the recombinant clones was carried out with Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). The sequencing was carried out by the dideoxy chain-stopping method of Sanger et al. (1977, Proceedings of the National Academies of Sciences U.S.A., 74:5463-5467) with modifications according to Zimmermann et al. (1990, Nucleic Acids Research, 18:1067). The "RR dRhodamin Terminator Cycle Sequencing Kit" from PE Applied Biosystems (Product No. 403044, Weiterstadt, Germany) was used. The separation by gel electrophoresis and analysis of the sequencing reaction were carried out in a "Rotiphoresis NF Acrylamide/Bisacrylamide" Gel (29:1) (Product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencer from PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained were then processed using the Staden program package (1986, Nucleic Acids Research, 14:217-231) version 97-0. The individual sequences of the pZero1 derivatives were assembled to a continuous contig. The computer-assisted coding region analysis were prepared

with the XNIP program (Staden, 1986, Nucleic Acids Research 14:217-231). Further analyses were carried out with the "BLAST search program" (Altschul et al., 1997, Nucleic Acids Research 25:3389-3402), against the non-redundant
5 databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The resulting nucleotide sequence is shown in SEQ ID No. 4. Analysis of the nucleotide sequence showed an open reading frame of 1737 base pairs, which was called the poxB gene.
10 The poxB gene codes for a polypeptide of 579 amino acids (SEQ ID NO. 5).

Example 10

Preparation of an integration vector for integration
mutagenesis of the poxB gene

15 From the strain ATCC 13032, chromosomal DNA was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)). On the basis of the sequence of the poxB gene known for C. glutamicum from Example 9, the following oligonucleotides were chosen for the polymerase chain
20 reaction:

poxBint1:

5' TGC GAG ATG GTG AAT GGT GG 3'

poxBint2:

5' GCA TGA GGC AAC GCA TTA GC 3'

25 The primers shown were synthesized by MWG Biotech (Ebersberg, Germany) and the PCR reaction was carried out by the standard PCR method of Innis et al. (PCR protocols. A guide to methods and applications, 1990, Academic Press) with Pwo-Polymerase from Boehringer. With the aid of the
30 polymerase chain reaction, a DNA fragment approx. 0.9 kb in size was isolated, this carrying an internal fragment of the poxB gene and being shown in SEQ ID No. 6.

The amplified DNA fragment was ligated with the TOPO TA Cloning Kit from Invitrogen Corporation (Carlsbad, CA, USA; Catalogue Number K4500-01) in the vector pCR2.1-TOPO (Mead et al. (1991) Bio/Technology 9:657-663). The E. coli Stamm DH5 α was then electroporated with the ligation batch (Hanahan, In: DNA cloning. A practical approach. Vol.I. IRL-Press, Oxford, Washington DC, USA, 1985). Selection for plasmid-carrying cells was made by plating out the transformation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), which had been supplemented with 25 mg/l kanamycin. Plasmid DNA was isolated from a transformant with the aid of the QIAprep Spin Miniprep Kit from Qiagen and checked by restriction with the restriction enzyme EcoRI and subsequent agarose gel electrophoresis (0.8%). The plasmid was called pCR2.1poxBint (Figure 4).

Plasmid pCR2.1poxBint has been deposited in the form of the strain Escherichia coli DH5 α /pCR2.1poxBint as DSM 13114 at the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) in accordance with the Budapest Treaty.

Example 11

Integration mutagenesis of the poxB gene in the lysine producer DSM 5715

The vector pCR2.1poxBint mentioned in Example 10 was electroporated by the electroporation method of Tauch et al. (FEMS Microbiological Letters, 123:343-347 (1994)) in Corynebacterium glutamicum DSM 5715. Strain DSM 5715 is an AEC-resistant lysine producer. The vector pCR2.1poxBint cannot replicate independently in DSM5715 and is retained in the cell only if it has integrated into the chromosome of DSM 5715. Selection of clones with pCR2.1poxBint

integrated into the chromosome was carried out by plating out the electroporation batch on LB agar (Sambrook et al., Molecular cloning: a laboratory manual. 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.), which
5 had been supplemented with 15 mg/l kanamycin. For detection of the integration, the poxBint fragment was labelled with the Dig hybridization kit from Boehringer by the method of "The DIG System Users Guide for Filter Hybridization" of Boehringer Mannheim GmbH (Mannheim, Germany, 1993).
10 Chromosomal DNA of a potential integrant was isolated by the method of Eikmanns et al. (Microbiology 140: 1817 - 1828 (1994)) and in each case cleaved with the restriction enzymes SallI, SacI and HindIII. The fragments formed were separated by agarose gel electrophoresis and hybridized at
15 68°C with the Dig hybridization kit from Boehringer. The plasmid pCR2.1poxBint mentioned in Example 9 had been inserted into the chromosome of DSM5715 within the chromosomal poxB gene. The strain was called DSM5715::pCR2.1poxBint.

20 Example 12

Effect of over-expression of the gnd gene with simultaneous elimination of the poxB gene on the preparation of lysine

12.1 Preparation of the strain

DSM5715::pCR2.1poxBint/pECgnd

25 The strain DSM5715::pCR2.1poxBint was transformed with the plasmid pECgnd using the electroporation method described by Liebl et al., (FEMS Microbiology Letters, 53:299-303 (1989)). Selection of the transformants took place on LBHIS agar comprising 18.5 g/l brain-heart infusion broth, 0.5 M
30 sorbitol, 5 g/l Bacto-tryptone, 2.5 g/l Bacto-yeast extract, 5 g/l NaCl and 18 g/l Bacto-agar, which had been supplemented with 5 mg/l tetracycline and 25 mg/l kanamycin. Incubation was carried out for 2 days at 33°C.

Plasmid DNA was isolated in each case from a transformant by conventional methods (Peters-Wendisch et al., 1998, Microbiology 144, 915 -927), cleaved with the restriction endonuclease AccI, and the plasmid was checked by
5 subsequent agarose gel electrophoresis. The strain obtained in this way was called DSM5715:pCR2.1poxBint/pECgnd.

12.2 Preparation of L-lysine

The *C. glutamicum* strain DSM5715::pCR2.1poxBint/pECgnd
10 obtained in Example 12.1 was cultured in a nutrient medium suitable for the production of lysine and the lysine content in the culture supernatant was determined.

For this, the strain was first incubated on an agar plate with the corresponding antibiotic (brain-heart agar with
15 tetracycline (5 mg/l) and kanamycin (25 mg/l)) for 24 hours at 33°C. The cultures of the comparison strains were supplemented according to their resistance to antibiotics. Starting from this agar plate culture, a preculture was seeded (10 ml medium in a 100 ml conical flask). The
20 complete medium CgIII was used as the medium for the preculture.

Medium Cg III

| | |
|------|---------|
| NaCl | 2.5 g/l |
|------|---------|

| | |
|---------------|--------|
| Bacto-Peptone | 10 g/l |
|---------------|--------|

| | |
|---------------------|--------|
| Bacto-Yeast extract | 10 g/l |
|---------------------|--------|

Glucose (autoclaved separately) 2% (w/v)

The pH was brought to pH 7.4

Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added to this. The preculture was incubated for 16 hours at 33°C at 240 rpm on a shaking machine. A main culture was seeded

from this preculture such that the initial OD (660nm) of the main culture was 0.1. Medium MM was used for the main culture.

Medium MM

| | |
|---|----------|
| CSL (corn steep liquor) | 5 g/l |
| MOPS (morpholinopropanesulfonic acid) | 20 g/l |
| Glucose (autoclaved separately) | 58 g/l |
| $(\text{NH}_4)_2\text{SO}_4$ | 25 g/l |
| KH_2PO_4 | 0.1 g/l |
| $\text{MgSO}_4 \cdot 7 \text{ H}_2\text{O}$ | 1.0 g/l |
| $\text{CaCl}_2 \cdot 2 \text{ H}_2\text{O}$ | 10 mg/l |
| $\text{FeSO}_4 \cdot 7 \text{ H}_2\text{O}$ | 10 mg/l |
| $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ | 5.0mg/l |
| Biotin (sterile-filtered) | 0.3 mg/l |
| Thiamine * HCl (sterile-filtered) | 0.2 mg/l |
| L-Leucine (sterile-filtered) | 0.1 g/l |
| CaCO_3 | 25 g/l |

5

The CSL, MOPS and the salt solution were brought to pH 7 with aqueous ammonia and autoclaved. The sterile substrate and vitamin solutions were then added, as well as the CaCO_3 autoclaved in the dry state.

Culturing is carried out in a 10 ml volume in a 100 ml conical flask with baffles. Tetracycline (5 mg/l) and kanamycin (25 mg/l) were added. Culturing was carried out at 33°C and 80% atmospheric humidity.

- 5 After 72 hours, the OD was determined at a measurement wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, München). The amount of lysine formed was determined with an amino acid analyzer from Eppendorf-BioTronik (Hamburg, Germany) by ion exchange chromatography
10 and post-column derivatization with ninhydrin detection.

The result of the experiment is shown in Table 3.

Table 3

| Strain | OD | L-Lysine HCl g/l |
|-----------------------------------|------|---------------------|
| DSM5715 | 10.8 | 16.0 |
| DSM5715/pECgnd | 7.6 | 16.5 |
| DSM5715::pCR2.1poxBint | 7.1 | 16.7 |
| DSM5715::pCR2.1poxBint/ pECgnd | 7.2 | 17.1 |

Patent claims

1. A process for the preparation of L-amino acids by fermentation of coryneform bacteria
5 w h i c h c o m p r i s e s
 carrying out the following steps:
 - a) fermentation of the desired L-amino acid-producing bacteria in which at least the gnd gene is amplified,
 - 10 b) concentration of the L-amino acid in the medium or in the cells of the bacteria and
 - c) isolation of the L-amino acid produced.
2. The process as claimed in claim 1,
 w h e r e i n
15 bacteria in which further genes of the biosynthesis pathway of the desired L-amino acid are additionally amplified, in particular over-expressed, are employed.
3. The process as claimed in claim 1,
 w h e r e i n
20 coryneform bacteria which prepare L-threonine, L-lysine, L-isoleucine or L-tryptophan are used.
4. The process as claimed in claim 3,
 w h e r e i n
 coryneform bacteria which prepare L-lysine are used.
- 25 5. A process for the fermentative preparation of L-lysine as claimed in claim 2,
 w h e r e i n
 in the coryneform microorganisms which in particular already produce L-lysine, one or more genes chosen from
30 the group consisting of
 - 5.1 the dapA gene which codes for dihydrodipicolinate synthase,

- 5.2 the lysC gene which codes for a feed back resistant aspartate kinase,
- 5.3 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
- 5 5.4 the pyc gene which codes for pyruvate carboxylase,
- 5.5 the tkt gene which codes for transketolase,
- 5.6 the zwf gene which codes for glucose 6-phosphate dehydrogenase,
- 10 5.7 the lysE gene which codes for lysine export,
- 5.8 the zwal gene
- 5.9 the eno gene which codes for enolase
- is or are amplified, in particular over-expressed, at the same time.
- 15 6. A process for the fermentative preparation of L-threonine as claimed in claim 2, wherein
- in the coryneform microorganisms which in particular already produce L-threonine, one or more genes chosen
- 20 from the group consisting of
- 6.1 the hom gene which codes for homoserine dehydrogenase or the hom^{dr} allele which codes for a "feed back resistant" homoserine dehydrogenase,
- 6.2 the gap gene which codes for glyceraldehyde 3-phosphate dehydrogenase,
- 25 6.3 the pyc gene which codes for pyruvate carboxylase,

6.4 the mqo gene which codes for malate:quinone
oxidoreductase,

6.5 the tkt gene which codes for transketolase,

6.6 the zwf gene which codes for glucose 6-phosphate
dehydrogenase,

6.7 the thrE gene which codes for threonine export,

6.8 the zwal gene,

6.9 the eno gene which codes for enolase

is or are amplified, in particular over-expressed, at
the same time.

7. The process as claimed in claim 2,

wherein

for the preparation of L-amino acids, in particular L-
lysine or L-threonine, bacteria in which one or more
genes chosen from the group consisting of,

7.1 the pck gene which codes for phosphoenol pyruvate
carboxykinase

7.2 the pgi gene which codes for glucose 6-phosphate
isomerase

7.3 the poxB gene which codes for pyruvate oxidase or

7.4 the zwa2 gene

is or are attenuated at the same time, are fermented.

8. The process as claimed in claims 2 to 6,

wherein

to achieve the amplification, the number of copies of
the genes or nucleotide sequences is increased by
transformation of the microorganisms with plasmid

vectors which carry these genes or nucleotide sequences.

9. The plasmid vector pEC-T18mob2 deposited under the designation DSM 13244 in *E. coli* K-12 DH5 α , shown in Figure 1.
10. A coryneform microorganism, in particular of the genus *Corynebacterium*, transformed by the introduction of the plasmid vector as claimed in claim 9, which additionally contains the *gnd* gene.

Figure 1: Map of the plasmid pEC-T18mob2

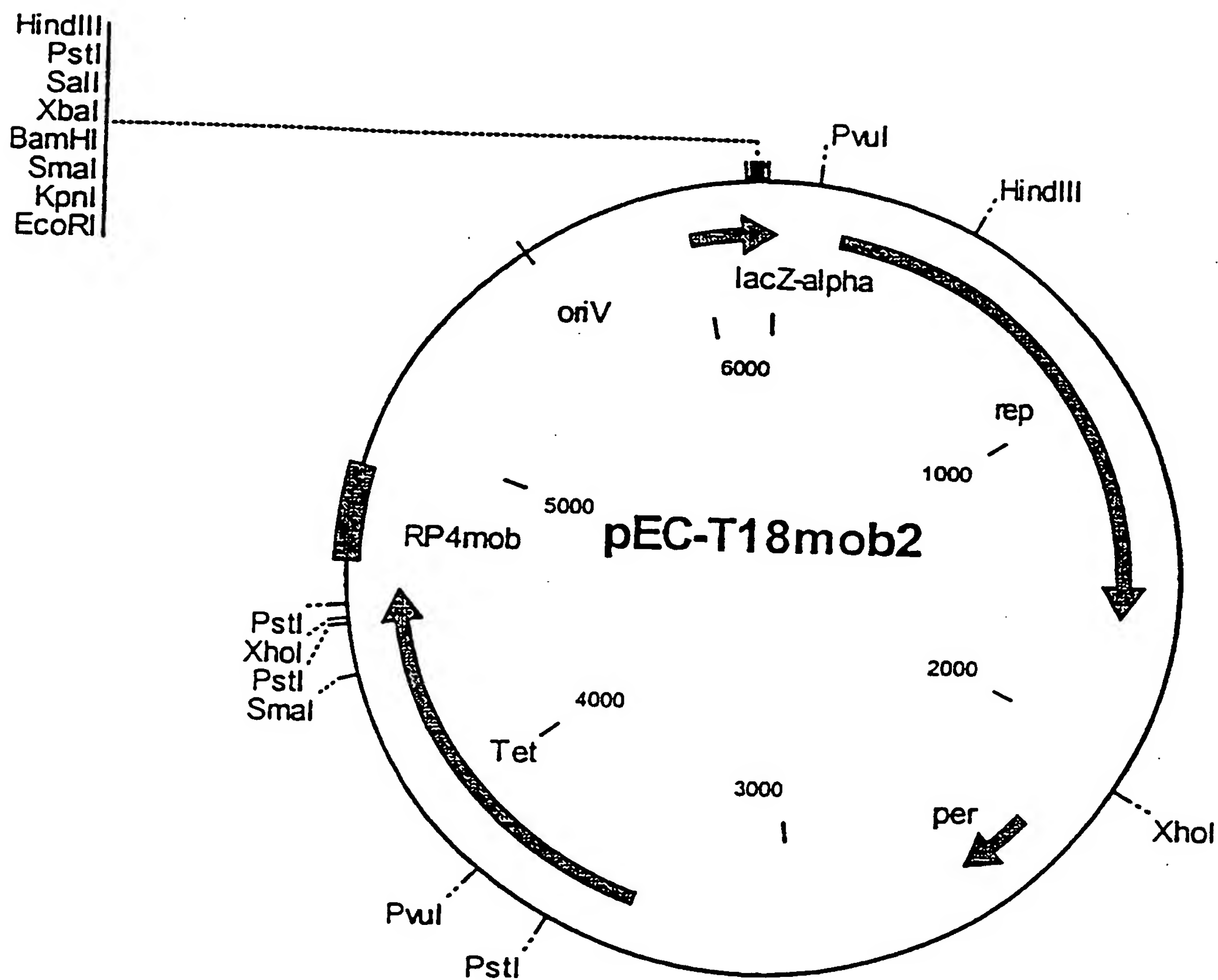


Figure 2: Map of the plasmid pECgnd

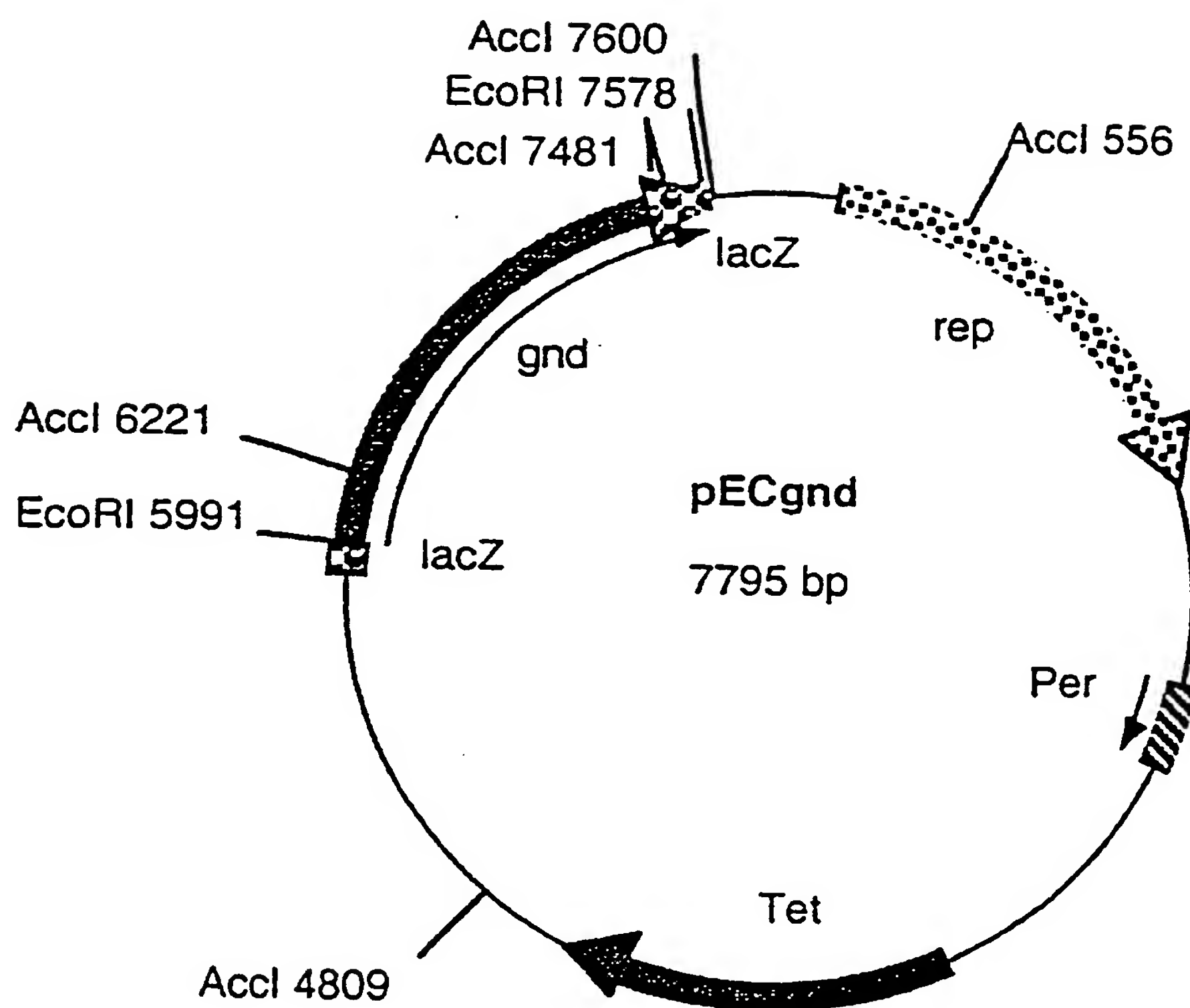


Figure 3: Map of the plasmid pBGNA

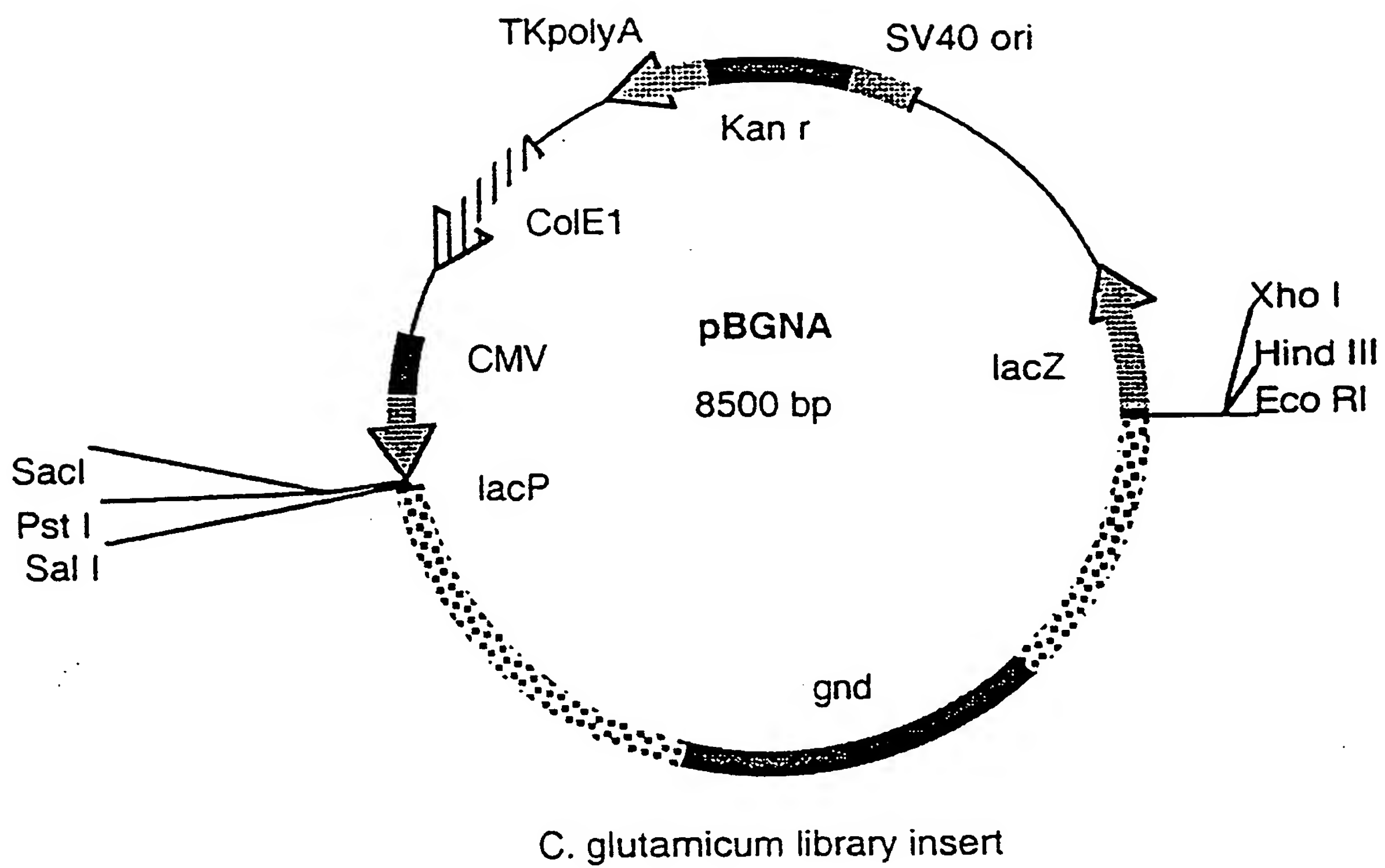
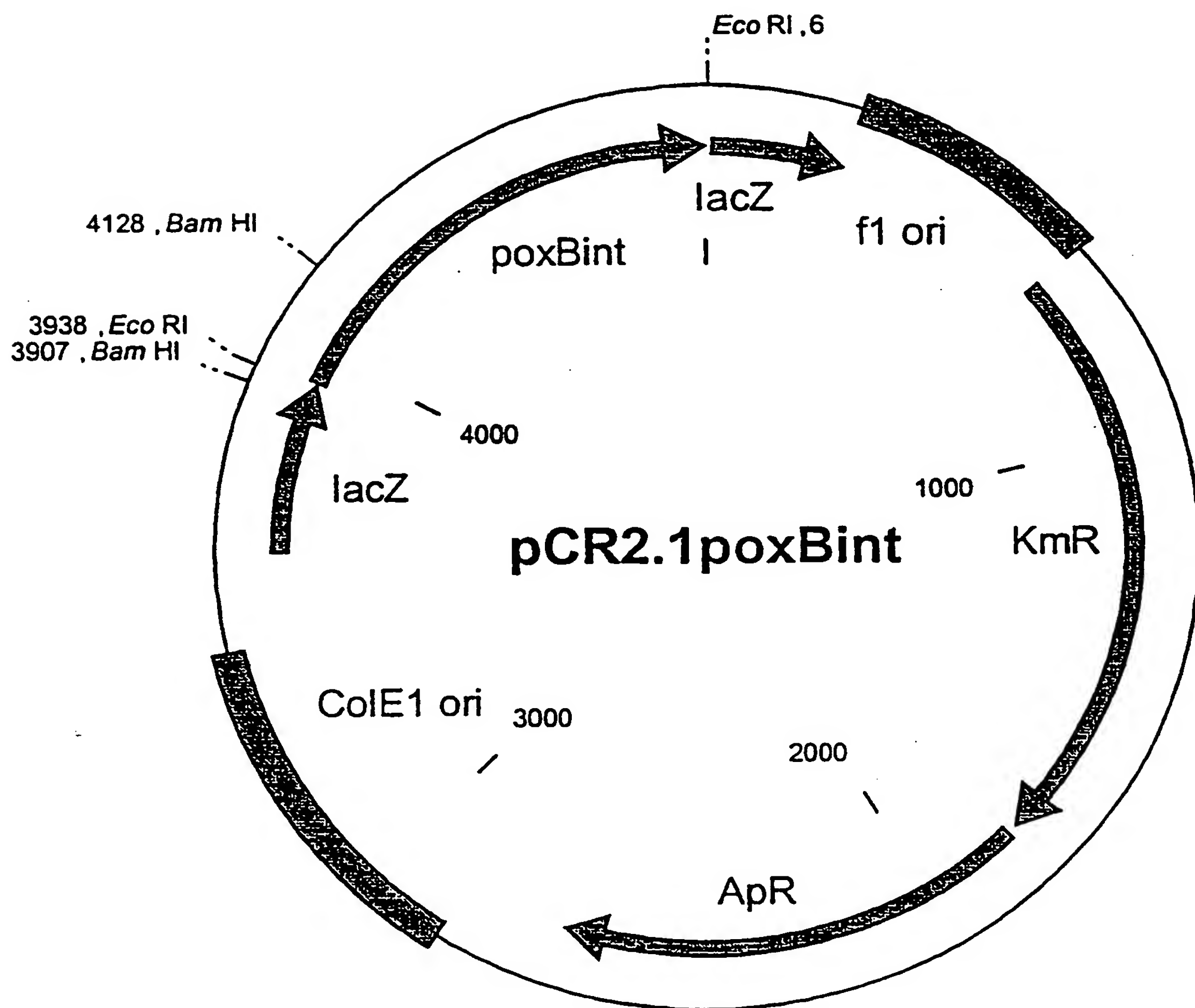


Figure 4: Map of the plasmid pCR2.1poxBint



SEQUENCE PROTOCOL

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 Degussa-Hüls AG

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 L-amino acids using coryneform bacteria.

10 <130> 990229 BT

<140>
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15 <160> 6

<170> PatentIn Ver. 2.1

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 55 ccactacccc caaatggtca caccttttag gccgattttg ctgacaccgg gct atg 476
 Met
 1

60 ccg tca agt acg atc aat aac atg act aat gga gat aat ctc gca cag 524
 Pro Ser Ser Thr Ile Asn Asn Met Thr Asn Gly Asp Asn Leu Ala Gln
 5 10 15

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| | Ile Gly Val Val Gly Leu Ala Val Met Gly Ser Asn Leu Ala Arg Asn | |
| | 20 25 30 | |
| 5 | ttc gcc cgc aac ggc aac act gtc gct gtc tac aac cgc agc act gac | 620 |
| | Phe Ala Arg Asn Gly Asn Thr Val Ala Val Tyr Asn Arg Ser Thr Asp | |
| | 35 40 45 | |
| 10 | aaa acc gac aag ctc atc gcc gat cac ggc tcc gaa ggc aac ttc atc | 668 |
| | Lys Thr Asp Lys Leu Ile Ala Asp His Gly Ser Glu Gly Asn Phe Ile | |
| | 50 55 60 65 | |
| 15 | cct tct gca acc gtc gaa gag ttc gta gca tcc ctg gaa aag cca cgc | 716 |
| | Pro Ser Ala Thr Val Glu Glu Phe Val Ala Ser Leu Glu Lys Pro Arg | |
| | 70 75 80 | |
| 20 | cgc gcc atc atc atg gtt cag gct ggt aac gcc acc gac gca gtc atc | 764 |
| | Arg Ala Ile Ile Met Val Gln Ala Gly Asn Ala Thr Asp Ala Val Ile | |
| | 85 90 95 | |
| 25 | aac cag ctg gca gat gcc atg gac gaa ggc gac atc atc atc gac ggc | 812 |
| | Asn Gln Leu Ala Asp Ala Met Asp Glu Gly Asp Ile Ile Ile Asp Gly | |
| | 100 105 110 | |
| 30 | ggc aac gcc ctc tac acc gac acc att cgt cgc gag aag gaa atc tcc | 860 |
| | Gly Asn Ala Leu Tyr Thr Asp Thr Ile Arg Arg Glu Lys Glu Ile Ser | |
| | 115 120 125 | |
| 35 | gca cgc ggt ctc cac ttc gtc ggt gct ggt atc tcc ggc ggc gaa gaa | 908 |
| | Ala Arg Gly Leu His Phe Val Gly Ala Gly Ile Ser Gly Gly Glu Glu | |
| | 130 135 140 145 | |
| 40 | ggc gca ctc aac ggc cca tcc atc atg cct ggt ggc cca gca aag tcc | 956 |
| | Gly Ala Leu Asn Gly Pro Ser Ile Met Pro Gly Gly Pro Ala Lys Ser | |
| | 150 155 160 | |
| 45 | tac gag tcc ctc gga cca ctg ctt gag tcc atc gct gcc aac gtt gac | 1004 |
| | Tyr Glu Ser Leu Gly Pro Leu Leu Glu Ser Ile Ala Ala Asn Val Asp | |
| | 165 170 175 | |
| 50 | ggc acc cca tgt gtc acc cac atc ggc cca gac ggc gcc ggc cac ttc | 1052 |
| | Gly Thr Pro Cys Val Thr His Ile Gly Pro Asp Gly Ala Gly His Phe | |
| | 180 185 190 | |
| 55 | gtc aag atg gtc cac aac ggc atc gag tac gcc gac atg cag gtc atc | 1100 |
| | Val Lys Met Val His Asn Gly Ile Glu Tyr Ala Asp Met Gln Val Ile | |
| | 195 200 205 | |
| 60 | ggc gag gca tac cac ctt ctg ccc tac gca gca ggc atg cag cca gct | 1148 |
| | Gly Glu Ala Tyr His Leu Pro Tyr Ala Ala Gly Met Gln Pro Ala | |
| | 210 215 220 225 | |
| 65 | gaa atc gct gag gtt ttc aag gaa tgg aac gca ggc gac ctg gat tcc | 1196 |
| | Glu Ile Ala Glu Val Phe Lys Glu Trp Asn Ala Gly Asp Leu Asp Ser | |
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| 70 | tac ctc atc gaa atc acc gca gag gtt ctc tcc cag gtg gat gct gaa | 1244 |
| | Tyr Leu Ile Glu Ile Thr Ala Glu Val Leu Ser Gln Val Asp Ala Glu | |
| | 245 250 255 | |
| 75 | acc ggc aag cca cta atc gac gtc atc gtt gac gct gca ggt cag aag | 1292 |
| | Thr Gly Lys Pro Leu Ile Asp Val Ile Val Asp Ala Ala Gly Gln Lys | |
| | 260 265 270 | |

| | | |
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| | ggc acc ggc aag tgg act gtc aag gct gct ctt gat ctg ggt att gct | 1340 |
| | Gly Thr Gly Lys Trp Thr Val Lys Ala Ala Leu Asp Leu Gly Ile Ala | |
| | 275 280 285 | |
| 5 | acc acc ggc atc ggc gaa cgt gtt ttc gca cgt gca ctc tcc ggc gca | 1388 |
| | Thr Thr Gly Ile Gly Glu Arg Val Phe Ala Arg Ala Leu Ser Gly Ala | |
| | 290 295 300 305 | |
| 10 | acc agc cag cgc gct gca gca cag ggc aac cta cct gca ggt gtc ctc | 1436 |
| | Thr Ser Gln Arg Ala Ala Ala Gln Gly Asn Leu Pro Ala Gly Val Leu | |
| | 310 315 320 | |
| 15 | acc gat ctg gaa gca ctt ggc gtg gac aag gca cag ttc gtc gaa gga | 1484 |
| | Thr Asp Leu Glu Ala Leu Gly Val Asp Lys Ala Gln Phe Val Glu Gly | |
| | 325 330 335 | |
| 20 | ctt cgc cgt gca ctg tac gca tcc aag ctt gtt gct tac gca cag ggc | 1532 |
| | Leu Arg Arg Ala Leu Tyr Ala Ser Lys Leu Val Ala Tyr Ala Gln Gly | |
| | 340 345 350 | |
| 25 | ttc gac gag atc aag gct ggc tcc gac gag aac aac tgg gac gtt gac | 1580 |
| | Phe Asp Glu Ile Lys Ala Gly Ser Asp Glu Asn Asn Trp Asp Val Asp | |
| | 355 360 365 | |
| 30 | cct cgc gac ctc gct acc atc tgg cgc ggc ggc tgc atc att cgc gct | 1628 |
| | Pro Arg Asp Leu Ala Thr Ile Trp Arg Gly Gly Cys Ile Ile Arg Ala | |
| | 370 375 380 385 | |
| 35 | aag ttc ctc aac cgc atc gtc gaa gca tac gat gca aac gct gaa ctt | 1676 |
| | Lys Phe Leu Asn Arg Ile Val Glu Ala Tyr Asp Ala Asn Ala Glu Leu | |
| | 390 395 400 | |
| 40 | gag tcc ctg ctg ctc gat cct tac ttc aag agc gag ctc ggc gac ctc | 1724 |
| | Glu Ser Leu Leu Leu Asp Pro Tyr Phe Lys Ser Glu Leu Gly Asp Leu | |
| | 405 410 415 | |
| 45 | atc gat tca tgg cgt cgc gtg att gtc acc gcc acc cag ctt ggc ctg | 1772 |
| | Ile Asp Ser Trp Arg Arg Val Ile Val Thr Ala Thr Gln Leu Gly Leu | |
| | 420 425 430 | |
| 50 | cca atc cca gtg ttc gct tcc tcc ctg tcc tac tac gac agc ctg cgt | 1820 |
| | Pro Ile Pro Val Phe Ala Ser Ser Leu Ser Tyr Tyr Asp Ser Leu Arg | |
| | 435 440 445 | |
| 55 | gca gag cgt ctg cca gca gcc ctg atc cac tagtgctcgac ctgcaggcgc | 1870 |
| | Ala Glu Arg Leu Pro Ala Ala Leu Ile His | |
| | 450 455 | |
| 60 | gcgagctcca gcttttggtc ccttttagtga gggttaattt cgagcttggc gtaatcaagg | 1930 |
| | tcatagctgt ttctgtgtg aaattggtat ccgctcaciaa ttccacaciaa tatacgagcc | 1990 |
| | ggaagtataa agtgtaaagc ctgggggtgcc taatgagtga gctaactcac agtaattgcg | 2050 |
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| | gcctaccgcc catttgctgc aatgggggcgg agttgttacg acattttgga aagtcccgtt | 2170 |
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| | gtgagtcaaaa ccgctatcca cgcccattga tgtactgcca aaaccgcatc accatggtaa | 2290 |

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 35 40 45

20 Asp Lys Thr Asp Lys Leu Ile Ala Asp His Gly Ser Glu Gly Asn Phe
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Ile Pro Ser Ala Thr Val Glu Glu Phe Val Ala Ser Leu Glu Lys Pro
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Ile Asn Gln Leu Ala Asp Ala Met Asp Glu Gly Asp Ile Ile Ile Asp
 100 105 110

30 Gly Gly Asn Ala Leu Tyr Thr Asp Thr Ile Arg Arg Glu Lys Glu Ile
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35 Ser Ala Arg Gly Leu His Phe Val Gly Ala Gly Ile Ser Gly Gly Glu
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Glu Gly Ala Leu Asn Gly Pro Ser Ile Met Pro Gly Gly Pro Ala Lys
 145 150 155 160

40 Ser Tyr Glu Ser Leu Gly Pro Leu Leu Glu Ser Ile Ala Ala Asn Val
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Asp Gly Thr Pro Cys Val Thr His Ile Gly Pro Asp Gly Ala Gly His
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45 Phe Val Lys Met Val His Asn Gly Ile Glu Tyr Ala Asp Met Gln Val
 195 200 205

50 Ile Gly Glu Ala Tyr His Leu Leu Pro Tyr Ala Ala Gly Met Gln Pro
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Ala Glu Ile Ala Glu Val Phe Lys Glu Trp Asn Ala Gly Asp Leu Asp
 225 230 235 240

55 Ser Tyr Leu Ile Glu Ile Thr Ala Glu Val Leu Ser Gln Val Asp Ala
 245 250 255

Glu Thr Gly Lys Pro Leu Ile Asp Val Ile Val Asp Ala Ala Gly Gln
 260 265 270

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 275 280 285

Ala Thr Thr Gly Ile Gly Glu Arg Val Phe Ala Arg Ala Leu Ser Gly
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 Leu Thr Asp Leu Glu Ala Leu Gly Val Asp Lys Ala Gln Phe Val Glu
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 10 Gly Leu Arg Arg Ala Leu Tyr Ala Ser Lys Leu Val Ala Tyr Ala Gln
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 Gly Phe Asp Glu Ile Lys Ala Gly Ser Asp Glu Asn Asn Trp Asp Val
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 15 Asp Pro Arg Asp Leu Ala Thr Ile Trp Arg Gly Gly Cys Ile Ile Arg
 370 375 380
 20 Ala Lys Phe Leu Asn Arg Ile Val Glu Ala Tyr Asp Ala Asn Ala Glu
 385 390 395 400
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 Met Ala His Ser Tyr Ala Glu Gln Leu
 1 5
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 Ile Asp Thr Leu Glu Ala Gln Gly Val Lys Arg Ile Tyr Gly Leu Val
 10 15 20 25

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| | ggt gac agc ctt aat ccg atc gtg gat gct gtc cgc caa tca gat att | 449 |
| | Gly Asp Ser Leu Asn Pro Ile Val Asp Ala Val Arg Gln Ser Asp Ile | |
| | 30 35 40 | |
| 5 | gag tgg gtg cac gtt cga aat gag gaa gcg gcg gcg ttt gca gcc ggt | 497 |
| | Glu Trp Val His Val Arg Asn Glu Glu Ala Ala Ala Phe Ala Ala Gly | |
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| | Ala Glu Ser Leu Ile Thr Gly Glu Leu Ala Val Cys Ala Ala Ser Cys | |
| | 60 65 70 | |
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| | Gly Pro Gly Asn Thr His Leu Ile Gln Gly Leu Tyr Asp Ser His Arg | |
| | 75 80 85 | |
| 20 | aat ggt gcg aag gtg ttg gcc atc gct agc cat att ccg agt gcc cag | 641 |
| | Asn Gly Ala Lys Val Leu Ala Ile Ala Ser His Ile Pro Ser Ala Gln | |
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| 25 | att ggt tcg acg ttc ttc cag gaa acg cat ccg gag att ttg ttt aag | 689 |
| | Ile Gly Ser Thr Phe Phe Gln Glu Thr His Pro Glu Ile Leu Phe Lys | |
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| 30 | gaa tgc tct ggt tac tgc gag atg gtg aat ggt ggt gag cag ggt gaa | 737 |
| | Glu Cys Ser Gly Tyr Cys Glu Met Val Asn Gly Gly Glu Gln Gly Glu | |
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| | Arg Ile Leu His His Ala Ile Gln Ser Thr Met Ala Gly Lys Gly Val | |
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| | Ser Val Val Val Ile Pro Gly Asp Ile Ala Lys Glu Asp Ala Gly Asp | |
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| 45 | ggt act tat tcc aat tcc act att tct tct ggc act cct gtg gtg ttc | 881 |
| | Gly Thr Tyr Ser Asn Ser Thr Ile Ser Ser Gly Thr Pro Val Val Phe | |
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| 50 | ccg gat cct act gag gct gca gcg ctg gtg gag gcg att aac aac gct | 929 |
| | Pro Asp Pro Thr Glu Ala Ala Ala Leu Val Glu Ala Ile Asn Asn Ala | |
| | 190 195 200 | |
| 55 | aag tct gtc act ttg ttc tgc ggt gcg ggc gtg aag aat gct cgc gcg | 977 |
| | Lys Ser Val Thr Leu Phe Cys Gly Ala Gly Val Lys Asn Ala Arg Ala | |
| | 205 210 215 | |
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| | Gln Val Leu Glu Leu Ala Glu Lys Ile Lys Ser Pro Ile Gly His Ala | |
| | 220 225 230 | |
| 65 | ctg ggt ggt aag cag tac atc cag cat gag aat ccg ttt gag gtc ggc | 1073 |
| | Leu Gly Gly Lys Gln Tyr Ile Gln His Glu Asn Pro Phe Glu Val Gly | |
| | 235 240 245 | |
| 70 | atg tct ggc ctg ctt ggt tac ggc gcc tgc gtg gat gcg tcc aat gag | 1121 |
| | Met Ser Gly Leu Leu Gly Tyr Gly Ala Cys Val Asp Ala Ser Asn Glu | |
| | 250 255 260 265 | |
| 75 | gcg gat ctg ctg att cta ttg ggt acg gat ttc cct tat tct gat ttc | 1169 |
| | Ala Asp Leu Leu Ile Leu Leu Gly Thr Asp Phe Pro Tyr Ser Asp Phe | |

| | 270 | | | | | | | | 275 | | | | | 280 | | | | | |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|------|--|--|
| 5 | ctt | cct | aaa | gac | aac | gtt | gcc | cag | gtg | gat | atc | aac | ggt | gcg | cac | att | 1217 | | |
| | Leu | Pro | Lys | Asp | Asn | Val | Ala | Gln | Val | Asp | Ile | Asn | Gly | Ala | His | Ile | | | |
| | | | | 285 | | | | | 290 | | | | | 295 | | | | | |
| 10 | ggt | cga | cgt | acc | acg | gtg | aag | tat | ccg | gtg | acc | ggt | gat | gtt | gct | gca | 1265 | | |
| | Gly | Arg | Arg | Thr | Thr | Val | Lys | Tyr | Pro | Val | Thr | Gly | Asp | Val | Ala | Ala | | | |
| | | | 300 | | | | | 305 | | | | | 310 | | | | | | |
| 15 | aca | atc | gaa | aat | att | ttg | cct | cat | gtg | aag | gaa | aaa | aca | gat | cgt | tcc | 1313 | | |
| | Thr | Ile | Glu | Asn | Ile | Leu | Pro | His | Val | Lys | Glu | Lys | Thr | Asp | Arg | Ser | | | |
| | | 315 | | | | | 320 | | | | | 325 | | | | | | | |
| 20 | ttc | ctt | gat | cgg | atg | ctc | aag | gca | cac | gag | cgt | aag | ttg | agc | tcg | gtg | 1361 | | |
| | Phe | Leu | Asp | Arg | Met | Leu | Lys | Ala | His | Glu | Arg | Lys | Leu | Ser | Ser | Val | | | |
| | | 330 | | | | 335 | | | | | 340 | | | | | 345 | | | |
| 25 | gta | gag | acg | tac | aca | cat | aac | gtc | gag | aag | cat | gtg | cct | att | cac | cct | 1409 | | |
| | Val | Glu | Thr | Tyr | Thr | His | Asn | Val | Glu | Lys | His | Val | Pro | Ile | His | Pro | | | |
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| 30 | gaa | tac | gtt | gcc | tct | att | ttg | aac | gag | ctg | gcg | gat | aag | gat | gcg | gtg | 1457 | | |
| | Glu | Tyr | Val | Ala | Ser | Ile | Leu | Asn | Glu | Leu | Ala | Asp | Lys | Asp | Ala | Val | | | |
| | | | | 365 | | | | | 370 | | | | | 375 | | | | | |
| 35 | ttt | act | gtg | gat | acc | ggc | atg | tgc | aat | gtg | tgg | cat | gcg | agg | tac | atc | 1505 | | |
| | Phe | Thr | Val | Asp | Thr | Gly | Met | Cys | Asn | Val | Trp | His | Ala | Arg | Tyr | Ile | | | |
| | | | 380 | | | | 385 | | | | | | 390 | | | | | | |
| 40 | gag | aat | ccg | gag | gga | acg | cgc | gac | ttt | gtg | ggt | tca | ttc | cgc | cac | ggc | 1553 | | |
| | Glu | Asn | Pro | Glu | Gly | Thr | Arg | Asp | Phe | Val | Gly | Ser | Phe | Arg | His | Gly | | | |
| | | 395 | | | | | 400 | | | | | 405 | | | | | | | |
| 45 | acg | atg | gct | aat | gcg | ttg | cct | cat | gcg | att | ggt | gcg | caa | agt | gtt | gat | 1601 | | |
| | Thr | Met | Ala | Asn | Ala | Leu | Pro | His | Ala | Ile | Gly | Ala | Gln | Ser | Val | Asp | | | |
| | | 410 | | | | 415 | | | | | 420 | | | | | 425 | | | |
| 50 | cga | aac | cgc | cag | gtg | atc | gcg | atg | tgt | ggc | gat | ggt | ggt | ttg | ggc | atg | 1649 | | |
| | Arg | Asn | Arg | Gln | Val | Ile | Ala | Met | Cys | Gly | Asp | Gly | Gly | Leu | Gly | Met | | | |
| | | | | 430 | | | | | | 435 | | | | | 440 | | | | |
| 55 | ctg | ctg | ggt | gag | ctt | ctg | acc | gtt | aag | ctg | cac | caa | ctt | ccg | ctg | aag | 1697 | | |
| | Leu | Leu | Gly | Glu | Leu | Leu | Thr | Val | Lys | Leu | His | Gln | Leu | Pro | Leu | Lys | | | |
| | | | | 445 | | | | | 450 | | | | | 455 | | | | | |
| 60 | gct | gtg | gtg | ttt | aac | aac | agt | tct | ttg | ggc | atg | gtg | aag | ttg | gag | atg | 1745 | | |
| | Ala | Val | Val | Phe | Asn | Asn | Ser | Ser | Leu | Gly | Met | Val | Lys | Leu | Glu | Met | | | |
| | | | 460 | | | | | 465 | | | | | 470 | | | | | | |
| 65 | ctc | gtg | gag | gga | cag | cca | gaa | ttt | ggt | act | gac | cat | gag | gaa | gtg | aat | 1793 | | |
| | Leu | Val | Glu | Gly | Gln | Pro | Glu | Phe | Gly | Thr | Asp | His | Glu | Glu | Val | Asn | | | |
| | | 475 | | | | | 480 | | | | | 485 | | | | | | | |
| 70 | ttc | gca | gag | att | gcg | gcg | gct | gcg | ggt | atc | aaa | tcg | gta | cgc | atc | acc | 1841 | | |
| | Phe | Ala | Glu | Ile | Ala | Ala | Ala | Ala | Gly | Ile | Lys | Ser | Val | Arg | Ile | Thr | | | |
| | | 490 | | | | 495 | | | | | 500 | | | | | 505 | | | |
| 75 | gat | ccg | aag | aaa | gtt | cgc | gag | cag | cta | gct | gag | gca | ttg | gca | tat | cct | 1889 | | |
| | Asp | Pro | Lys | Lys | Val | Arg | Glu | Gln | Leu | Ala | Glu | Ala | Leu | Ala | Tyr | Pro | | | |
| | | | | 510 | | | | | | 515 | | | | | 520 | | | | |
| 80 | gga | cct | gta | ctg | atc | gat | atc | gtc | acg | gat | cct | aat | gcg | ctg | tcg | atc | 1937 | | |

Gly Pro Val Leu Ile Asp Ile Val Thr Asp Pro Asn Ala Leu Ser Ile
 525 530 535

5 cca cca acc atc acg tgg gaa cag gtc atg gga ttc agc aag gcg gcc 1985
 Pro Pro Thr Ile Thr Trp Glu Gln Val Met Gly Phe Ser Lys Ala Ala
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10 acc cga acc gtc ttt ggt gga gga gta gga gcg atg atc gat ctg gcc 2033
 Thr Arg Thr Val Phe Gly Gly Gly Val Gly Ala Met Ile Asp Leu Ala
 555 560 565

15 cgt tcg aac ata agg aat att cct act cca tgatgattga tacacctgct 2083
 Arg Ser Asn Ile Arg Asn Ile Pro Thr Pro
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40 Glu Leu Ala Val Cys Ala Ala Ser Cys Gly Pro Gly Asn Thr His Leu
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Ile Gln Gly Leu Tyr Asp Ser His Arg Asn Gly Ala Lys Val Leu Ala
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45 Ile Ala Ser His Ile Pro Ser Ala Gln Ile Gly Ser Thr Phe Phe Gln
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Glu Thr His Pro Glu Ile Leu Phe Lys Glu Cys Ser Gly Tyr Cys Glu
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55 Gln Ser Thr Met Ala Gly Lys Gly Val Ser Val Val Val Ile Pro Gly
 145 150 155 160

Asp Ile Ala Lys Glu Asp Ala Gly Asp Gly Thr Tyr Ser Asn Ser Thr
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60 Ile Ser Ser Gly Thr Pro Val Val Phe Pro Asp Pro Thr Glu Ala Ala
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Ala Leu Val Glu Ala Ile Asn Asn Ala Lys Ser Val Thr Leu Phe Cys

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|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| | Gly | Ala | Gly | Val | Lys | Asn | Ala | Arg | Ala | Gln | Val | Leu | Glu | Leu | Ala | Glu |
| | 210 | | | | | | 215 | | | | | 220 | | | | |
| 5 | Lys | Ile | Lys | Ser | Pro | Ile | Gly | His | Ala | Leu | Gly | Gly | Lys | Gln | Tyr | Ile |
| | 225 | | | | | 230 | | | | | 235 | | | | | 240 |
| | Gln | His | Glu | Asn | Pro | Phe | Glu | Val | Gly | Met | Ser | Gly | Leu | Leu | Gly | Tyr |
| 10 | | | | | 245 | | | | | 250 | | | | | 255 | |
| | Gly | Ala | Cys | Val | Asp | Ala | Ser | Asn | Glu | Ala | Asp | Leu | Leu | Ile | Leu | Leu |
| | | | | 260 | | | | | 265 | | | | | 270 | | |
| 15 | Gly | Thr | Asp | Phe | Pro | Tyr | Ser | Asp | Phe | Leu | Pro | Lys | Asp | Asn | Val | Ala |
| | | | 275 | | | | | 280 | | | | | 285 | | | |
| | Gln | Val | Asp | Ile | Asn | Gly | Ala | His | Ile | Gly | Arg | Arg | Thr | Thr | Val | Lys |
| 20 | | 290 | | | | | 295 | | | | | 300 | | | | |
| | Tyr | Pro | Val | Thr | Gly | Asp | Val | Ala | Ala | Thr | Ile | Glu | Asn | Ile | Leu | Pro |
| | 305 | | | | | 310 | | | | | 315 | | | | | 320 |
| | His | Val | Lys | Glu | Lys | Thr | Asp | Arg | Ser | Phe | Leu | Asp | Arg | Met | Leu | Lys |
| 25 | | | | | 325 | | | | | 330 | | | | | 335 | |
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| | | | | 340 | | | | | 345 | | | | | 350 | | |
| 30 | Val | Glu | Lys | His | Val | Pro | Ile | His | Pro | Glu | Tyr | Val | Ala | Ser | Ile | Leu |
| | | | 355 | | | | | 360 | | | | | 365 | | | |
| | Asn | Glu | Leu | Ala | Asp | Lys | Asp | Ala | Val | Phe | Thr | Val | Asp | Thr | Gly | Met |
| 35 | | 370 | | | | | 375 | | | | | 380 | | | | |
| | Cys | Asn | Val | Trp | His | Ala | Arg | Tyr | Ile | Glu | Asn | Pro | Glu | Gly | Thr | Arg |
| | 385 | | | | | 390 | | | | | 395 | | | | | 400 |
| | Asp | Phe | Val | Gly | Ser | Phe | Arg | His | Gly | Thr | Met | Ala | Asn | Ala | Leu | Pro |
| 40 | | | | | 405 | | | | | 410 | | | | | 415 | |
| | His | Ala | Ile | Gly | Ala | Gln | Ser | Val | Asp | Arg | Asn | Arg | Gln | Val | Ile | Ala |
| | | | | 420 | | | | | 425 | | | | | 430 | | |
| 45 | Met | Cys | Gly | Asp | Gly | Gly | Leu | Gly | Met | Leu | Leu | Gly | Glu | Leu | Leu | Thr |
| | | | 435 | | | | | 440 | | | | | 445 | | | |
| | Val | Lys | Leu | His | Gln | Leu | Pro | Leu | Lys | Ala | Val | Val | Phe | Asn | Asn | Ser |
| 50 | | 450 | | | | | 455 | | | | | 460 | | | | |
| | Ser | Leu | Gly | Met | Val | Lys | Leu | Glu | Met | Leu | Val | Glu | Gly | Gln | Pro | Glu |
| | 465 | | | | | 470 | | | | | 475 | | | | | 480 |
| | Phe | Gly | Thr | Asp | His | Glu | Glu | Val | Asn | Phe | Ala | Glu | Ile | Ala | Ala | Ala |
| 55 | | | | | 485 | | | | | 490 | | | | | 495 | |
| | Ala | Gly | Ile | Lys | Ser | Val | Arg | Ile | Thr | Asp | Pro | Lys | Lys | Val | Arg | Glu |
| | | | | 500 | | | | | 505 | | | | | 510 | | |
| 60 | Gln | Leu | Ala | Glu | Ala | Leu | Ala | Tyr | Pro | Gly | Pro | Val | Leu | Ile | Asp | Ile |
| | | | 515 | | | | | 520 | | | | | 525 | | | |
| | Val | Thr | Asp | Pro | Asn | Ala | Leu | Ser | Ile | Pro | Pro | Thr | Ile | Thr | Trp | Glu |

530 535 540

Gln Val Met Gly Phe Ser Lys Ala Ala Thr Arg Thr Val Phe Gly Gly
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5 Gly Val Gly Ala Met Ile Asp Leu Ala Arg Ser Asn Ile Arg Asn Ile
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Pro Thr Pro

10

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 <211> 875
 <212> DNA
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20 <400> 6

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35